How minor blood collection differences can cause major issues for bioanalytical methods

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Abstract

Novel aspects

Minor blood collection differences can cause major differences in responses within analytical methods.

Introduction

As stated in the latest ICH guideline (M10) on bioanalytical method validation and study sample analysis (effective since January 21, 2023): "The matrix used for bioanalytical method validation should be the same as the matrix of the study samples, including anticoagulants and additives." However, even when these conditions are met, unexpected differences in matrices can occur due to the fact that they generally come from different commercial sources and from (pre-)clinical studies for which detailed information is not always available.

Methods

The issues were observed with an industry standard drug quantification ligand binding assay and a functional neutralizing antibody (NAb) cell-based assay.

For the drug quantification assay, a sandwich ELISA format was used, where the drug is captured using a specific biotinylated monoclonal antibody onto a streptavidin coated plate and detected using a second specific sulfo-TAG™-labeled monoclonal antibody. For detection, an electrical potential causes light emission from the sulfo-TAG™ labels that are bound. The ECL signal is measured using a MESO™ Sector S600 plate reader (Meso Scale Discovery).

For the functional NAb assay, a cell-based assay was used; cell growth is induced using the drug (a cytokine glycoprotein). When drug-neutralizing antibodies are present, they prevent the drug from inducing cell growth. Cell growth is measured using the CellTiter-Glo® Luminescent Cell Viability Assay.

Results

For the drug quantification assay, unexpected diverging matrix selectivity results were obtained during method development with different commercial plasma lots prepared using the sodium citrate anticoagulant. Eventually, it was found that the diverging results were caused by different sodium citrate concentrations used for plasma preparation; the existence of different sodium citrate concentration for plasma isolation was not suspected as clinical protocols do not always specify which sodium citrate concentration is used and collection tubes with sodium citrate are sometimes only available at one concentration, depending on the supplier. When different sodium citrate concentrations (3.8% and 3.2%) were compared, results confirmed the significant effect sodium citrate concentrations even though it was not initially suspected as a cause for observed differences. To mitigate the issue and validate the assay using 3.2% sodium citrate plasma, the MRD from 1/10 which produced acceptable matrix selectivity results with the plasma prepared with 3.8% sodium citrate had to be increased to 1/50 to produce acceptable matrix selectivity results.

For the functional NAb assay, initial testing compared serum from healthy and cancer subjects since the assay was being developed for analysis of clinical study samples from both populations. However, observed cell proliferation variability was greater within healthy subjects than between healthy and cancer subjects. After careful investigation and documentation review, it was found that serum from healthy subjects which had been collected in bags caused cell proliferation to be low and variable and explained the observed differences. The use of serum collected in bags would have created very difficult conditions for a successful method validation. To resolve the issue, serum samples collected in serum SST tubes were used for method validation; a sensitive assay with a cut point more representative of the expected clinical study population was obtained. The assay was successfully used to analyze samples from multiple clinical trials.

Conclusions

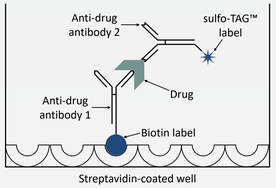
Although guidance documents are clear in that matrices used during validation should be the same as the study samples (type, anticoagulant, additives), additional minor differences should be considered when selecting commercials matrices for method validation; commercial sources should be asked to provide as much information as possible about the matrices being purchased because it should not be assumed that commercial matrices will be equivalent to the same matrices collected for clinical studies.

Methods

Quantitative assay

The method used to perform drug measurements is an industry standard sandwich ELISA using the MSD platform in 96-well plates: the biotinylated capture antibody, sample with drug and sulfo TAG™ labeled detection antibody are each sequentially added after washes, and detection is performed using the MSD 600 instrument; Figure 1 illustrates the capture and detection of the drug with the biotinylated monoclonal antibody and sulfo-TAG™-labeled monoclonal antibody, respectively.

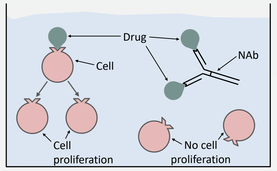
Figure 1
Quantitative assay



NAb assay

The method used to detect NAbs is an industry standard cell-based assay in 96-well plates: drug-induced cell growth (suspension cell line) is measured after adding heat-inactivated serum samples with a final MRD of 1/20 in cell culture media in the presence of drug inducer and incubating for 2 days at 37°C, 5% CO_2. Cell viability is then measured using the CellTiter-Glo® assay. Results are normalized to the signal of the viability control sample (serum pool without NAbs and with drug inducer). Figure 2 illustrates the drug-induced cell growth which can be inhibited when NAbs are present.

Figure 2
NAb assay

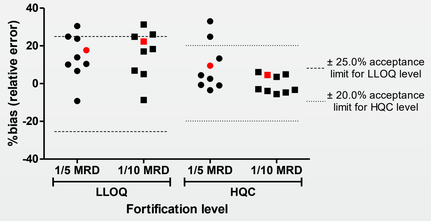


Results

Quantitative assay

During the development of a quantitative method measuring the concentration of the drug in plasma, different minimum required dilutions were tested to minimize matrix interference without sacrificing assay sensitivity. Results are presented in Figure 3.

Figure 3
Matrix selectivity results in plasma collected with 3.8% sodium citrate



Each symbol represents the result from one tested plasma lot; the red symbols illustrate data for the spike control samples in a pooled plasma lot (3.8% sodium citrate)

With similar acceptable results at the LLOQ level and significantly better results at the HQC level, the 1/10 MRD was selected to pursue assay development.

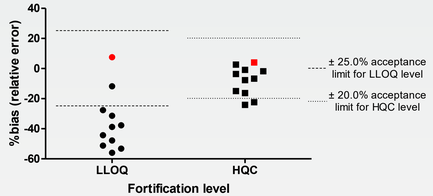
However, an eventual notification by the sponsor was provided stating that the plasma samples from the clinical study which would be analyzed using the method would be collected using 3.2% sodium citrate. After verification because the sodium citrate concentration had not been discussed before, it was confirmed that the initial matrix selectivity results were obtained with plasma collected using 3.8% sodium citrate instead of 3.2%. Although no issues were expected as:

- the matrix used throughout the method (standards, QC samples, spike control samples, etc.) was changed from plasma collected with 3.8% sodium citrate to plasma collected with 3.2% sodium citrate;
- the 1/10 MRD was maintained,

an additional matrix selectivity evaluation was still performed to confirm the absence of matrix effect of plasma collected with 3.2% sodium citrate. Results are presented in Figure 4.

Results (continued)

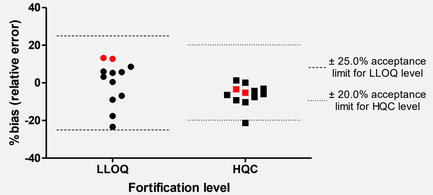
Figure 4
Matrix selectivity results in plasma collected with 3.2% sodium citrate (MRD 1/10)



Each symbol represents the result from one tested plasma lot; the red symbols illustrate data for the spike control samples in a pooled plasma lot (3.2% sodium citrate)

Unexpectedly, there was a significant matrix effect at the LLOQ level which had not been observed with plasma collected using sodium citrate 3.8%; the observed negative biases would not meet validation requirements. Further optimizations were therefore performed in an attempt to reduce the observed matrix effect. As presented in Figure 5, among the tested conditions, the increased MRD of 1/50 generated acceptable matrix selectivity results when using plasma collected using sodium citrate 3.2%.

Figure 5
Matrix selectivity results in plasma collected with 3.2% sodium citrate (MRD 1/50)



Each symbol represents the result from one tested plasma lot; the red symbols illustrate data for the spike control samples in a pooled plasma lot (3.2% sodium citrate)

With the MRD of 1/50, the method remained at the required assay sensitivity of 50 ng/mL, and the method was subsequently successfully validated.

NAb assay

During the development of a qualitative method to detect NAbs in serum, different types of serum were tested; the method was going to be used to detect NAbs in cancer patients, but if serum from "normal" subjects behaved the same way in the assay, it would have been preferred to prepared control samples due to its greater availability. Testing was performed using serum ordered from a commercial supplier; if divergences occurred, they were expected between serum from cancer patients and serum from "normal" subjects. "VC" in the following figures refers to the Viability Control (serum without NAbs) which would generate the maximum cell growth for this method.

As presented in Figure 6 and Figure 7, diverging results were obtained, but mainly between two batches of serum from "normal subjects" instead of between serum from cancer patients and from "normal subjects". Additionally:

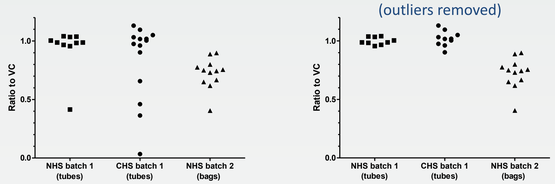
- Cell proliferation when in presence of NHS batch 2 samples was highly variable and generally much lower than in presence of NHS batch 1 or CHS batch 1 samples
- The box-plot outlier removal procedure efficiently removed outliers from NHS batch 1 and CHS batch 1, whereas it did not identify any outliers in NHS batch 2 due to its higher variability

The determination of a usable cut-point with a data distribution as seen with NHS batch 2 would therefore not be possible.

Results (continued)

Figure 6
Results from unfortified matrix lots

Figure 7
Results from unfortified matrix lots (outliers removed)



Each symbol represents the result from one tested serum lot; NHS refers to serum from "normal" subjects and CHS refers to serum from subjects with cancer

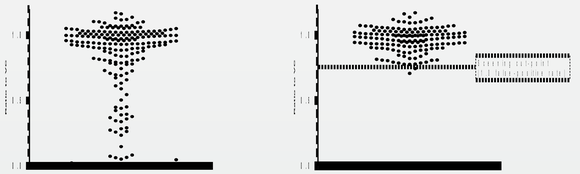
Communications with the commercial supplier to understand what the differences between the different serum batches led to the discovery that the collection method was not always the same although this was not specified in the different serum lot certificates of analysis:

- NHS batch 1: serum collected in tubes
- CHS batch 1: serum collected in tubes
- NHS batch 2: serum collected in bags

For the subsequent validation, serum collected in tubes was used. Cut-point results (4 batches of 52 serum lots tested by 2 analysts) obtained during method validation are presented in Figure 8 and Figure 9, and this cut-point was used for analysis of clinical study samples. Even using serum samples collected in tubes, the cut-point results show a high incidence of outliers which neutralize cell-growth non-specifically. For this reason, a confirmatory assay tier using a non-specific cell inducer was included for the detection of NAbs.

Figure 8
Results from validation cut-point runs (serum from cancer patients)

Figure 9
Results from validation cut-point runs (serum from cancer patients, outliers removed)



Each symbol represents one result from a tested serum lot (each of the 52 lots were tested 4 times)

Conclusions

Although guidance documents are clear in that matrices used during validation should be the same as the study samples (type, anticoagulant, additives), additional minor differences should be considered when selecting commercial matrices for method validation; ideally, commercial sources should be asked to provide as much information as possible about the matrices being purchased because it should not be assumed that commercial matrices will be equivalent to the same matrices collected for clinical studies.

Examples of minor differences in matrix results presented in this poster which can greatly affect include the use of different sodium citrate concentrations to collect plasma samples (3.2% versus 3.8%) and the use of different serum collection methods (in tubes versus in bags).